EFFECT OF TRIPTOLIDE ON PROLIFERATION AND APOPTOSIS OF ANGIOTENSIN II-INDUCED CARDIAC FIBROBLASTS *IN VITRO*: A PRELIMINARY STUDY

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Abstract

Background: The effect of triptolide (TPL) on cardiac fibroblasts (CFbs) and cardiac fibrosis remain unknown till now. This study was conducted to explore the effects of TPL on proliferation and apoptosis of angiotensin II (Ang II)-induced CFbs.

Materials and Methods: Ang II was used to promote proliferation of CFbs. Two dosages of TPL (10ng/ml and 100ng/ml) were chosen. MTT assay was used to detect cell survival rate *in vitro*. Flow cytometer was performed to analyze apoptosis of CFbs. Hydroxyproline concentration was detected with hydroxyproline assay kit. Quantitative real-time PCR was used to detect the expression of TGF-β1 and Smad3 mRNA.

Results: Ang II promoted CFbs proliferation significantly. Compared to Ang II group, TPL markedly reduced the viability of CFbs and its Hydroxyproline concentration ($P<0.05$). Besides, TPL can significantly promote apoptosis of CFbs ($P<0.05$). Furthermore, TPL reduced the expressions of TGF-β1 and Smad3 mRNA in Ang II-induced CFbs ($P<0.05$).

Conclusion: TPL can inhibit the proliferation of CFbs in rats by down-regulating TGF-β1/Smad3 signaling pathway. TPL might be a promising therapeutic drug for myocardial fibrosis.

Key words: Cardiac fibroblast; triptolide; proliferation; apoptosis; angiotensin

Introduction

Cardiac fibroblasts (CFbs), the highest cell population in the myocardium, play major roles in maintaining normal cardiac structure and function (Goldsmith et al., 2014). CFbs are of utmost importance for the pathogenesis of myocardial fibrosis. CFbs promote cardiac repair by controlling the synthesis and degradation of extracellular matrix (Dixon and Cunnington, 2011; Goldsmith et al., 2014). In the pathological status, some mechanical and chemical stimuli may promote the migration, proliferation and differentiation of CFbs, which may secrete excess amount of collagen fibers. As a result, cardiac fibrosis or myocardial scarring occurs and progresses gradually.

Myocardial fibrosis has already become one of the most difficult stages in the treatment of chronic heart failure (CHF) hence has prognostic relevance. Thus, how to ameliorate myocardial fibrosis is a current research topic in the field of CHF (Heymans et al., 2015). The mechanisms of CHF are diverse and complex (Yancy et al., 2013). Among the risk factors, immune-inflammatory activation was reported to be a cornerstone in the development and progression of myocardial fibrosis and CHF. Therefore, anti-inflammatory and immunomodulation therapy has been widely studied around the world and might be a promising strategy for the treatment of CHF in the future (Liu et al., 2015; Nymo et al., 2014).

Triptolide (TPL) is a diterpene triepoxide extracted from Tripterygium wilfordii Hook F, which has already been used in Traditional Chinese Medicine for thousands of years to treat immune-related diseases. It's a monomeric compound and has been reported to have immunosuppressive and anti-inflammatory properties (Li et al., 2014; Liu et al., 2015). Leonard et al. found that TPL can inhibit accumulation of inflammatory cells and may have therapeutic benefits for patients with obliterate airway disease (Leonard et al., 2002). However, whether TPL can inhibit the proliferation of CFbs and attenuate myocardial fibrosis in CHF rats are still uncertain. We hypothesized that TPL, as an immunosuppressive agent, could reverse myocardial fibrosis and inhibit CFbs proliferation in CHF rats.

Therefore, a series of experiments *in vivo* and *in vitro* were conducted to evaluate the effects of TPL on myocardial fibrosis in rats and on proliferation of CFbs in neonatal rats. In our previous study, we have demonstrated TPL significantly attenuated myocardial fibrosis and improved cardiac function through suppressing several signaling pathways in isoprenaline-induced cardiac remodeling rats (Liu et al., 2015). In this study, we assessed the effects of TPL on proliferation and apoptosis of angiotensin II (Ang II)-induced CFbs. This study revealed the effects of TPL on myocardial fibrosis at the cellular level.

Materials and methods
Chemicals and reagents

TPL ($C_{20}H_{24}O_6$, purity>98% by HPLC) was obtained from Gold Wheat Biotech Co., Ltd. (Shanghai, China). Ang II ($C_{50}H_{71}N_{13}O_{12}$, purity>98.0% by HPLC) was purchased from Meilun Biotech Co., Ltd. (Dalian, China). Dulbecco's modified eagle medium/Nutrient mixture F-12 (DMEM/F-12, 1:1) was purchased from Hyclone (Utah, USA). TPL was dissolved in dimethyl sulfoxide (DMSO), which was obtained from Hyclone (Utah, USA). Methylthiazolyldiphenyl-tetrazolium bromide assay kit (MTT), Annexin V-FITC apoptosis kit and hydroxyproline (HYP) testing kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TRIzol (Invitrogen, CA, USA), All-in-One TM first strand cDNA synthesis kit and All-in-One TM quantitative real-time polymerase chain reaction (PCR) mix (SYBR Green Method, GeneCopoeia, MD, USA) were also used. CO_2 incubator (HERAEUS, BB15, USA), quantitative real-time PCR (EPPENDORF, Mastercycler ep realplex[2], Germany) and flow cytometer (BECKMAN COULTER, COULTER EPICS XL, USA) were used.

Culture of rat CFbs

Primary CFbs of neonatal Wistar rats were obtained from Jiangyin CHI Scientific, Inc. (Jiangyin, China). The product number and verification number are No.66160-232 and No.20140520HXM. The primary CFbs were cultured in DMEM/F-12(1:1) containing 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 at 37℃. The nutrient medium was changed every 48 hrs. When the cells coated the bottom of the bottle, they will be sub-cultured in new culture dishes. The second and third generations of CFbs were chosen to use in this study.

Grouping and interventions

There were four groups set in this study: control (Con), Ang II (Ang), low-dose triptolide (LT) and high-dose triptolide group (HT). Con group was cultured in DMEM/F-12(1:1) only. Ang group was cultured in DMEM/F-12(1:1) with Ang II (10^{-7} mol/L). LT group was cultured in DMEM/F-12(1:1) with Ang II (10^{-7} mol/L) and TPL (10 ng/ml). HT group was cultured in DMEM/F^{-12}(1:1) with Ang II (10^{-7} mol/L) and TPL (100 ng/ml). The CFbs in these groups were all incubated under a humidified atmosphere of 5% CO_2 at 37℃ for 48 hours.

MTT assay

The viabilities of rat CFbs in different groups were calculated to evaluate the proliferation activity *in vitro* by using the MTT assay. The rat CFbs were harvested and seeded with a rate of 2000 cells per each hole of the 96-well plates at a density of 2×10^4 /mL during the logarithmic growth phase. They were cultured in DMEM/F-12(1:1) with 10% fetal calf serum under a humidified atmosphere of 5% CO_2 at 37℃ for 24 hours. Then, different dosages of drugs were given. The CFbs were further cultured in DMEM/F-12(1:1) with 1% fetal calf serum under the same circumstance. According to the instruction of MTT assay kit, 50μl of 1×MTT solution was added to each well. The CFbs were incubated in DMEM/F-12(1:1) with 1% fetal calf serum for an additional four hours. Then, 150μl DMSO was used to dissolve the MTT-formazan crystals. The optical density (OD) ($\lambda = 630$ nm) was obtained by using a microplate reader.

The corrected ODs were calculated by subtracting the OD of empty well. At last, the viability in each group was calculated according to the following equation: Viability = (Corrected OD of treated group/ Corrected OD of control group) × 100%.

HYP concentration

The rat CFbs were seeded in each well of the 24-well plates at a density of 5×10^5 /mL during the logarithmic growth phase. They were cultured in under a humidified atmosphere of 5% CO_2 at 37℃ for 24 hours. Then, different dosages of drugs were given. Finally, the CFbs were incubated in DMEM/F-12(1:1) with 1% fetal calf serum for an additional 72 hrs. The rat CFbs were harvested and the concentration of HYP was detected according to the assay kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Flow cytometer

Different dosages of drugs were added in each well when the CFbs were in logarithmic growth phase. After an additional 72 hrs incubation, the cells were harvested. Then, Annexinn V-FITC 5μl and PI 5μl were added in the tube for 10 min in a condition away from light. At last, the apoptotic CFbs were identified by flow cytometer and apoptotic rates were calculated by CXP 2.0 software.

Real-time PCR

Total RNA was extracted from CFbs by TRIzol reagent and reverse transcribed to cDNA by the All-in-One TM first strand cDNA synthesis kit. Two microliter cDNA were amplified by quantitative real-time PCR using the All-in-One TM quantitative real-time PCR mix. Beta-actin was used as an internal control gene to normalize the mRNA levels of transforming growth factor-β1 (TGF-β1) and drosophila mothers against decapentaplegic proteins-3 (Smad3). The sequences used in this study were as follows: TGF-β1(F) 5'-TGCTTCAGCTCCACAGAGAA-3', TGF-β1(R) 5'-TGGTTGTAGAGGGCAAGGAC-3', Smad3(F) 5'-GGCAGGATGTTTCCAGCTA-3',Smad3(R) 5'-GCAGTCC ACAGACCATGTCA-3', β-actin(F) 5'-AGGGAAATCGTGCGTGACAT-3', β-actin (R) 5'-GAACCGCTCATTGCCGATAG-3'. Each experiment was performed at least three times.

Statistical analysis

IBM SPSS software Version 20.0 for Windows (SPSS Inc., Chicago, USA) was used for the data analysis. The data were presented as mean ± standard deviation. Normality tests and homogeneity of variance tests were performed. One way ANOVA followed by LSD tests was used in the multiple comparisons of quantitative data. While

Kruskal-Wallis test was performed if non-normal distribution or heterogeneity of variance was found. A two-tale P<0.05 was considered to indicate a significant difference (Liu et al., 2015).

Results

Effects of TPL and Ang on proliferation of rat CFbs

The cultured CFbs in Con group were typical fibroblast morphology which were spindle-shaped or irregular triangle. The CFbs distributed evenly and tightly on the culture flask. In Ang group, the CFbs proliferated significantly, arranged closely and were in the shape of fusiform or square. The numbers of CFbs decreased markedly after the treatment of TPL. There were a few scattered dead cells in the culture flask (**Fig.1**).
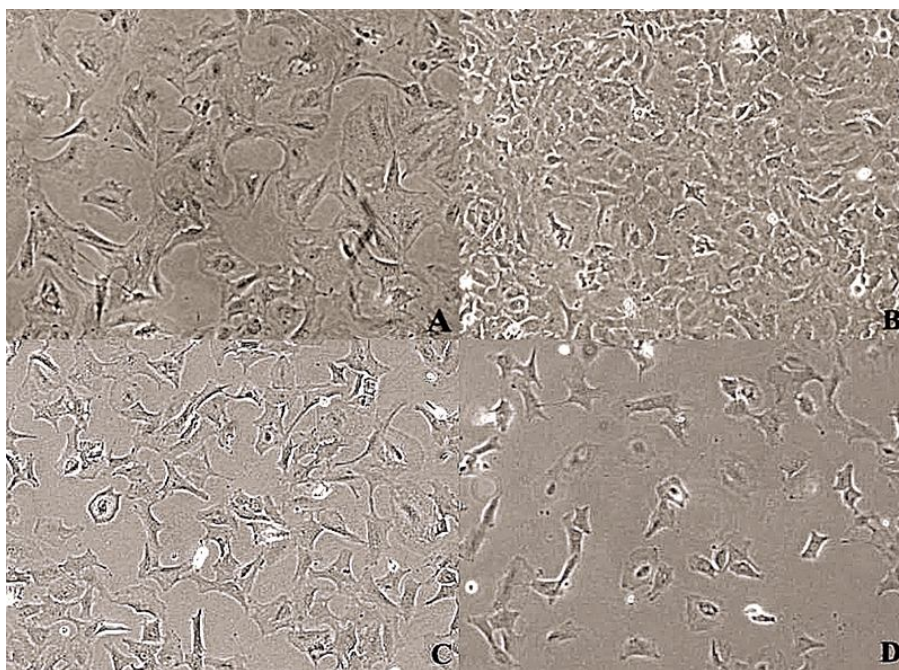

Figure 1: Morphological features of the CFbs in different groups (100×).

The cultured cells in Con group were typical fibroblast morphology and distributed evenly and tightly (A). The CFbs in Ang group proliferated significantly and were in the shape of fusiform or square (B). In the LT (C) and HT group (D), the numbers of CFbs were decreased markedly, especially in the HT group. Con=control; Ang=Angiotensin II; LT=low-dose triptolide; HT=high-dose triptolide group.

Effect of TPL on viability of rat CFbs

The viability of CFbs in Ang group was significantly higher than that of Con group (P=0.001). Compared to Ang group, the viability of CFbs was markedly reduced in HT group (3.24±1.58 vs. 1.23±0.34, P=0.006). No significant differences of viabilities were found between LT and Ang group (**Fig.2**).
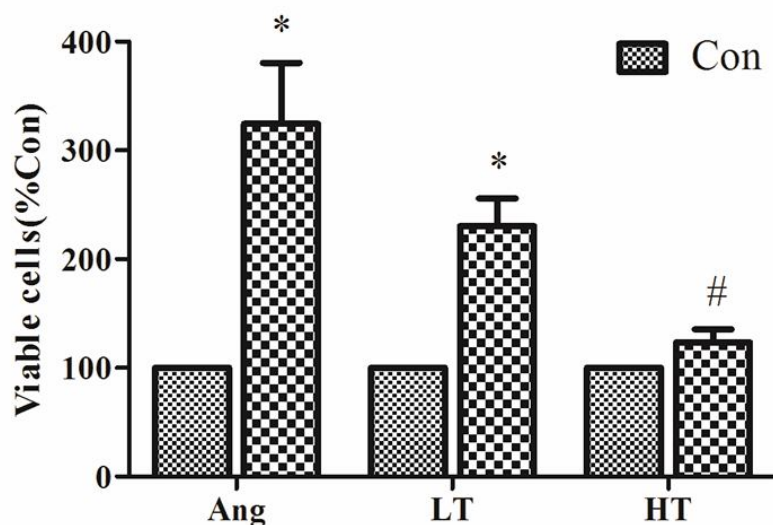
Figure 2: Effect of TPL on viability of CFbs (n=8).

Compared to Ang group, the viabilities of CFbs in LT and HT group decreased significantly. Con=control;

Ang=Angiotensin II; LT=low-dose triptolide; HT=high-dose triptolide group.

* P<0.05 vs. Con; # P<0.05 vs. Ang

Effect of TPL on HYP concentration in rat CFbs

The HYP concentration in Ang group was significantly higher than that of Con group (1.43±0.54 vs. 0.40±0.29, P=0.007). The HYP concentration in HT group was lower than that of Ang group (0.59±0.20 vs. 1.43±0.54, P=0.019) and LT group (0.59±0.20 vs. 1.27±0.30, P=0.047). No marked differences in the HYP concentration between LT and Ang group were found (**Fig.3**).
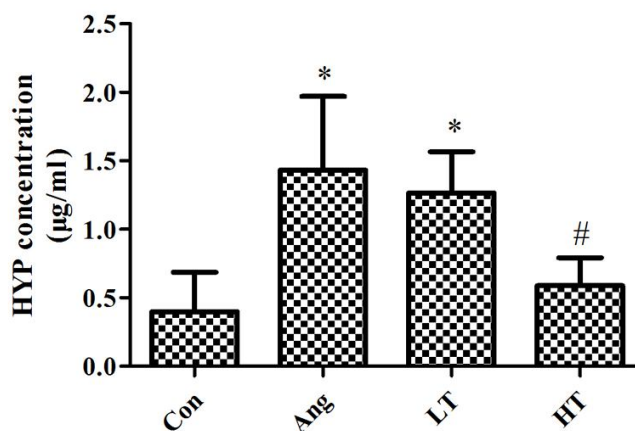

Figure 3: Effect of TPL on HYP concentration in CFbs (n=3).

Compared to Con group, Ang may increase the collagen concentration in CFbs. Compared to Ang group, the collagen concentrations in LT and HT group decreased significantly. HYP=hydroxyproline; Con=control; Ang=Angiotensin II; LT=low-dose triptolide; HT=high-dose triptolide group.

*P<0.05 vs. Con; #P<0.05 vs. Ang

Effect of TPL on apoptosis of rat CFbs

As shown in **Fig.4**, there was no noticeable difference in the apoptosis rate of CFbs between Ang and Con group (1.14 ± 0.52 vs. 1.09 ± 0.55, P>0.05). Compared to Ang group, the apoptosis rates of CFbs in LT group (3.00 ± 0.68 vs. 1.14 ± 0.52, P=0.012) and HT group (5.29 ± 0.97 vs. 1.14 ± 0.52, P<0.001) were significantly increased. Moreover, the apoptosis rate was higher in HT group than that of LT group P=0.004).
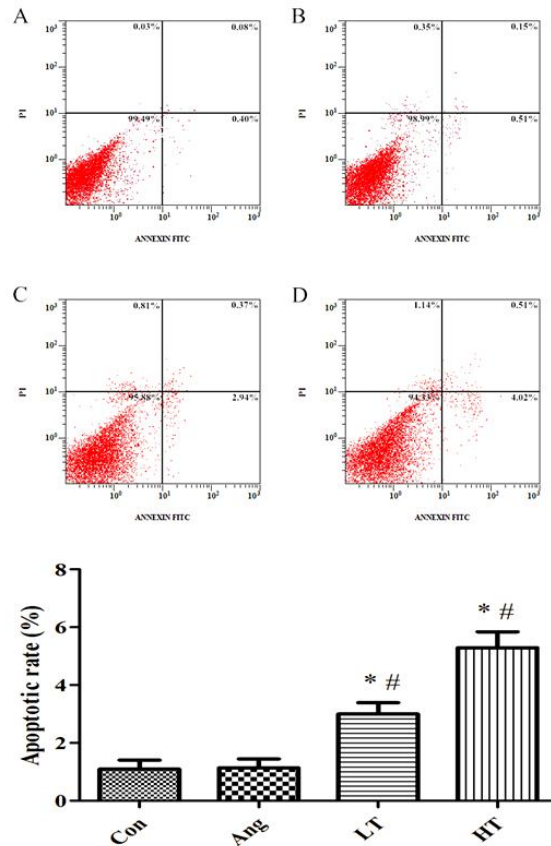

Figure 4: Effect of TPL on apoptosis of CFbs (n=3).
Compared to Ang group, TPL treatment significantly promoted apoptosis of CFbs, especially in HT group. A=Con (control); B=Ang (Angiotensin II); C=LT (low-dose triptolide); D=HT (high-dose triptolide group). *P<0.05 vs. Con; #P<0.05 vs. Ang

TPL down-regulates TGF-β1/Smad3 signaling in rat CFbs

As shown in **Fig.5**, In the HT group, the expressions of TGF-β1 mRNA (0.66 ± 0.15 vs. 1.36 ± 0.12, P<0.001) and Smad3 mRNA (0.25 ± 0.10 vs. 1.53 ± 0.14, P=0.022) were significantly lower than those of Ang group. Also, compared to Ang group, the expression of TGF-β1 mRNA was reduced in LT group (0.74 ± 0.09 vs. 1.36 ± 0.12, P=0.001). There was no statistical difference in the comparison of expressions of Smad3 mRNA between LT and Ang group (P>0.05).
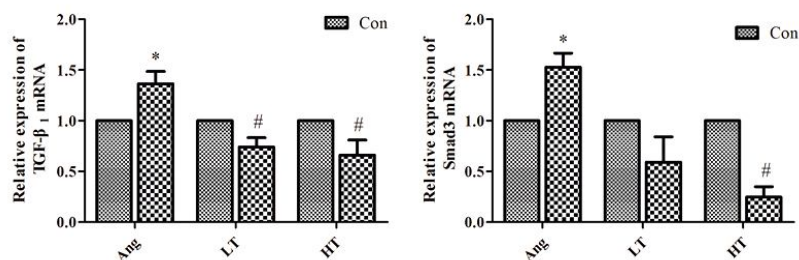
Figure 5: Effect of TPL on TGF-β1/Smad3 signaling in CFbs (n=3).
Compared to Ang group, the expressions of TGF-β1 mRNA reduced in HT and LT group. The expression of Smad3 mRNA significantly reduced in HT group. TGF-β1=transforming growth factor-β1; Smad3=drosophila mothers against decapentaplegic proteins-3; Con=control; Ang=Angiotensin II; LT=low-dose triptolide; HT=high-dose triptolide group.
[*]$P<0.05$ vs. Con; [#]$P<0.05$ vs. Ang

Discussion

In this study, we evaluated the effects of TPL on proliferation and apoptosis of Ang II-induced rat CFbs *in vitro*. Ang II can induce proliferation and increase the HYP concentration of CFbs significantly. TPL inhibited the Ang II-induced proliferation of CFbs from neonatal rats. Compared to Ang group, TPL markedly reduced the viability of CFbs in a dose dependent trend and the number of CFbs decreased markedly after the treatment of TPL. Besides, we firstly found that TPL can promote apoptosis of Ang II-induced CFbs, which may be an important mechanism of its antifibrotic effect to myocardial fibrosis in CHF rats. Moreover, TPL can also attenuate pro-fibrotic signaling in CFbs. Compared to Ang group, the HYP concentration and expressions of TGF-β1 mRNA and Smad3 mRNA were significantly lower in TPL treated CFbs. It is also the first report concerning the effect of TPL on TGF-β1/Smad3 pathway in CFbs.

It has been well known that Ang II is one of the most important vasoactive peptide in the renin-angiotensin-aldosterone system (Velez et al., 2012). When Ang II binds with its specific receptor, it can promote the secretion of aldosterone and cause water-sodium retention, which are essential in the development of myocardial fibrosis, hypertension and CHF. Ang II can also promote proliferation and differentiation of CFbs, increase the synthesis and excretion of fibrotic factors and collagens, resulting in the occurrence and progress of myocardial fibrosis (Yamazaki et al., 2012). Therefore, Ang II is often used to introduce CFbs proliferation in relevant cell experiments, including our present study.

TPL is a diterpene triepoxide and its molecular formula $C_{20}H_{24}O_6$. TPL is a monomeric compound extracted from a traditional Chinese herb Tripterygium wilfordii Hook F (Liu et al., 2015). It has been proved to have significant immunosuppressive and anti-inflammatory properties (Chen et al., 2015; Liu et al., 2015; Zhang et al., 2009). Tripterygium wilfordii was effective in halting the progress of graft coronary arteriosclerosis by inhibiting of the PDGF-A chain mRNA in cardiac graft (Hachida et al., 1999). It can also improve cardiac function and quality of life in rheumatoid arthritis patients by increasing peripheral blood B and T lymphocyte attenuator expression of CD19^{+} and CD24^{+} B cells and reducing B cell-mediated abnormal humoral immunity and oxidative stress damage (Cao et al., 2011; Sun et al., 2015). As for cell experiments, Lu et al. (Lu et al., 2008) found that TPL effectively reduced the expression of IL-18 at protein and gene levels and inhibited the bioactivity of IL-18 and nuclear factor-κB in rheumatoid arthritis synovial fibroblasts. But the effects of TPL on CFbs were barely reported. To the best of our knowledge, only Xu et al (Xu et al., 2008) had reported that TPL reduced the CFbs proliferation and the synthesis of pro α1 (I) collagen in a dose-dependent way (Xu et al., 2008). Then they drew a conclusion that TPL might attenuate myocardial fibrosis through inhibiting the proliferation and collagen synthesis of CFbs. However, the evidence is insufficient to support this conclusion because no animal experiment has been conducted. As is well known today,

TGF-β1/Smad3 signaling pathway is one of the dominant pathways in fibrosis (Wang et al., 2013). TGF-β1 has been considered as an attractive therapeutic target in the treatment of fibrotic diseases (Lee et al., 2014). The activation of this pathway may promote CFbs differentiation, increase the extracellular matrix protein deposition and connective tissue growth factor production (Liu et al., 2015; Zhang et al., 2013). However, these factors were not detected in Xu's study.

Therefore, based on the experience of previous studies, some improvements and complements were achieved. In our animal experiment, TPL has shown its antifibrotic effect in isoprenaline-induced CHF and cardiac fibrosis rats (Liu et al., 2015). In this *in vitro* study, the expressions of TGF-β1 and Smad3 mRNA of CFbs were significantly reduced by TPL, indicating that TPL may affect cardiac fibrosis via down-regulating TGF-β1/Smad3 signaling. Besides, we also found that TPL can inhibit the proliferation and reduce the HYP concentration in Ang II-induced CFbs, which were similar to the previous report (Xu et al., 2008). Furthermore, the pro-apoptic effect of TPL has been reported in lymphoblastic leukemia cells, cardiomyocytes and airway smooth muscle cells (Chen et al., 2015; Zhou et al., 2015). In this study, the effect of TPL on CFbs apoptosis was firstly determined. The results indicated that TPL can promote apoptosis of Ang II-induced CFbs in a dose dependent way, which might also play an important role in the antifibrotic effect of TPL in the treatment of myocardial fibrosis. There are some limitations of our study. Firstly, this is a basic research. The results and conclusions cannot be expanded to the clinical aspects relating human beings. TPL cannot be prescribed for the CHF patients yet. Further clinical trials are needed. Secondly, the number of tested groups was rather small. Only two different dosages were set in this study. And there was only one intervention-time point (48 h) set in this study because it's a preliminary experiment. Besides, no inflammatory factors and apoptosis-related proteins were included in this experiment. Therefore, we will test more intervention groups and detect more indexes in the following studies.

In conclusion, TPL can inhibit proliferation, promote apoptosis and reduce the expressions of TGF-β1 and Smad3 mRNA in Ang II-induced CFbs. TPL might be a promising therapeutic drug for myocardial fibrosis. Further studies are needed to explore the potential mechanism and clinical efficiency before it is widely used in CHF patients.

Disclosure of conflicts of interest

None

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